昆 虫 学 报 *Acta Entomologica Sinica*, Februray 2009, 52(2): 121 – 125 ISSN 0454-6296

EAG and behavioral responses of the large black chafer, *Holotrichia parallela* (Coleoptera: Scarabaeidae) to its sex pheromone

ZHOU Li-Mei[2], JU Qian[1], QU Ming-Jing[1,*], ZHAO Zhi-Qiang[1], DONG Shuang-Lin[3], HAN Zhao-Jun[3], YU Shan-Lin[1]

(1. Shandong Peanut Research Institute, Qingdao, Shandong 266100, China;
2. College of Plant Protection, Yunnan Agricultural University, Kunming, 650201, China;
3. Department of Plant Protection, Nanjing Agricultural University, Nanjing 210095, China)

Abstract: A method measuring EAG (electroantennogram) response in lamellate antennae of *Holotrichia parallela* Motschulsky was established in this study. A higher value of EAG was got when lamellae were separated from each other by insect pin. With the concentration of 30 ng · μL^{-1}, the EAG value of male antennae reached the maximum when the volume ratio of L-isoleucine methyl ester (LIME) to (R)-(-)-linalool was 7∶1, which was almost equal to the EAG value of the crude extracts from 25 sex glands. The male antennae were treated with different concentrations of stimulative reagent with the fixed volume ratio (7∶1) of LIME to (R)-(-)-linalool. The highest EAG value of male antennae was found when the concentration was 30 ng · μL^{-1}. The results of olfactory response further confirmed that male *H. parallela* was attracted with the selection efficiency of 93.3% when the volume ratio of LIME to (R)-(-)-linalool was 7∶1. So it can be deduced that the sex pheromone of *Holotrichia parallela* in Shandong province were the mixture of LIME and (R)-(-)-linalool with the ratio of 7∶1. The results may serve as basis for developing the techniques of sex pheromone attraction to control *H. parallela*.

Key words: *Holotrichia parallela*; sex pheromone; EAG; olfactory response; L-isoleucine methyl ester; (R)-(-)-linalool

1 INTRODUCTION

The loss of peanut production caused by grub is the most gravest of all underground insects. Forty species have been found to live on peanuts at the least. *Holotrichia parallela* is dominant among those species which damage peanuts, which is responsible for no less than 70 percent of the damage caused by the underground pests. The peanut yields were markedly reduced by 20 – 30 percent or more, sometimes no yield, and the peanut quality decreased obviously such as high rate of no-core peanuts without core and with empty-shell. *H. parallela* has been the main factor that constrains the peanut production. According to the successive investigation for five years in Shandong, Henan, Anhui, Hebei and Liaoning provinces, the damage caused by *H. parallela* was more than 1.5 – 10^8 kg annually (Yu *et al.*, 2007). Therefore, it is urgent to control the grub in peanut production.

The grub has evolved high resistance against pesticides for high-toxicity and high-residue pesticides used for long period, the quantity of predatory natural enemies also decreased seriously, and the chemical pollution is more severe than ever (Yao *et al.*, 2004). The sex lure could be used to predict the beetle occurrence and attract the adults of *H. parallela* to eliminate them in group, and then decrease the grub damage in the next year. Leal (1992) and Leal *et al.* (1993) has reported that L-isoleucine methyl ester (LIME) and (R)-(-)-linalool were separated and identified as the two constituents in the sex pheromone of *H. parallela*. Generally the composition of sex pheromone differs in ratio or constituents in the different area, and even in the same pest (Dong and Du, 2002; Zhang and Liu, 2003). So it is essential to study the large black beetle in China and the composition of sex pheromone, which will make a good foundation for

基金项目: 农业部引进国际先进农业科学技术项目(2006-G24); 公益性行业科研专项(nyhyzx07-014); 青岛市公共领域支撑项目(08-2-1-25-nsh)
作者简介: 周丽梅, 女, 1982 年生, 云南靖江人, 硕士研究生, 主要从事昆虫化学生态学研究, Tel.: 0532-87628320; E-mail: zlm060606@126.com
* 通讯作者 Author for correspondence, Tel.: 13455277580; E-mail: cygnet@njau.edu.cn
收稿日期 Received: 2008-09-16; 接受日期 Accepted: 2009-01-20

the application of the sex lure further.

Both EAG response and olfactory response are important methods to identify sex pheromones. It is difficult to carry out EAG experiment with antenna of the beetle because it is short and lamellate. The value recorded in EAG was low in the previous report, which is lack of descriptions in detail (Zhang *et al.*, 2006). In this study, we established an EAG method on lamellate antennae and determined the EAG response of *H. parallela* male adults to different ratios of compositions of sex pheromone, and olfactory response was also used to identify the EAG results further here. The results will be helpful for sex lure application in the control of *H. parallela*.

2 MATERIALS AND METHODS

2.1 Insects

The beetles were captured from the farm of Shandong Peanut Research Institute in Wangcheng, Shandong province. They were reared in a box containing soil and fresh elm leaves with the temperature of 25℃, relative humidity of 80%, and photoperiod of 12L:12D.

2.2 Antennae preparation

The antennae were treated with three methods: (1) The tip (1 mm) of lamellae was cut after the whole antenna was cut from the base; (2) The whole section of lamellae was cut after the whole antenna was cut from the base; (3) The three lamellae were dispersed from each other by a pin after their tips of edge were cut a bit (about 1 mm) (Fig. 1 shows that the tips of edge were cut along the line).

Each side of the antenna was stuck to the two metal electrodes by conductive adhesive. The level of EAG responses was compared among the three methods for preparing the antenna. The best method was applied to carry out the experiments subsequently.

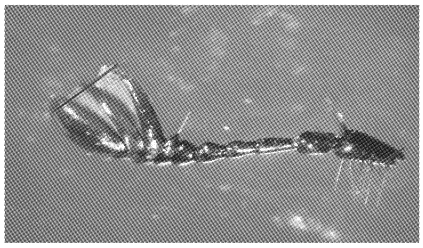

Fig. 1 Treatments to the antennae of *Holotrichia parallela*

2.3 EAG determination

10 μL sex lure was applied on a piece of filter paper (25 mm × 8 mm) by micro-syringe. After volatilization for 1 min, the paper can be used and the EAG response was recorded. The stimulus at 500 ms lasts 30 s. Both the persistence airflow and the stimulus airflow were 4 mL/s. Only one of the two antennas of each male adult was used. Six antennae were studied in every treatment with three repetitions. The dichloromethane was used as control, and the control and the sample were tested alternately. The value of EAG was recorded by measuring the scale of the downside peak and the final value of the test should subtract the mean of the two control values. The EAG instrument was composed of tiny controller INR-5, data gathering IDAC-4, odor stimulus set cs-05 and EAG result output systems. The whole equipment was made by Syntech Company of Holland.

2.4 EAG sample

L-isoleucine methyl ester (LIME) and (R)-(-)-linalool (purity >98%) were supplied by Sigma Company. All of them were dissolved to different concentrations (300, 30, 3, 0.3, 0.03 and 0.003 ng/μL) with vaporized dichloromethane. Six different ratios (3:1, 4:1, 5:1, 6:1, 7:1, 8:1) of LIME and (R)-(-)-linalool were made of the solution (30 ng/μL). Vaporized dichloromethane was used as control.

Collection of sex gland supernatant: Before copulating, the female beetles were captured and used to make sex gland supernatant. Firstly, the abdominal tips of 25 female were excised from their body quickly with forceps, and then the glands were separately washed with dichloromethane for 2 h at 4℃. After that, the supernatant was purified with filter paper (0.22 μm).

2.5 Olfactory response

Olfactory experiment was conducted in a dark room with the temperature 27 ± 1℃. The speed of airflow in two tubes is controlled at 500 mL/min, and ten vivid insects were used in each treatment. The beetles in two tubes were checked after 5 min. Both the control and treatment were taken in two tubes alternately with three repeats, and the tubes were washed with acetone. The response rate, rate of selective response and selection coefficient were calculated by formula listed as follows:

Response rate = $[(A+B)/C] \times 100\%$;

Rate of selective response = $[A/(A+B)] \times 100\%$

Selection coefficient = $[(A-B)/(A+B)]$

Where A represents beetles in sex lure tube; B represents beetles in control tube; C represents all the tested beetles.

Y-type olfactory instrument was composed of the ACO-006 air condenser (Guangdong Risheng Group Co., Ltd), LZB-4 glass rotor airflow controller

(Changzhou Shuanghuan Thermo-technical Instrument Co., Ltd), 250 mL T-colander, thribble, Y-tube (base tube 20 cm, two arms 12 cm, tube diameter 3.5 cm, the angle of two arms is 45°)

2.6 Statistical analysis of data

The data were analyzed with Duncan's test by DPS software.

3 RESULTS

3.1 EAG response of different treatments to antennae of *Holotrichia parallela*

Table 1 showed that the EAG response in method 3 was stronger than those in method 1 and 2 when lamellate antennae were separated from each other with pin. The value was almost equal to that of the crude extract. So method 3 was most favorable for EAG experiment of lamellate antennae and used in the following study.

Table 1 The EAG value of different treatments to antennae of *Holotrichia parallela*

Treatments	EAG response (mV)
Method 1	2.669 ±0.383 aA
Method 2	1.206 ±0.181 aA
Method 3	13.005 ±2.770 bB
Crude extract	12.444 ±2.330 bB

The data in the table are mean ± *SD*, and those followed by different letters are significantly different at the level of 0.05 and 0.01 by Duncan's multiple range test. The same below.

3.2 The EAG response of *Holotrichia parallela* to LIME and (R)-(-)-linalool

The results indicate that the beetles had response to LIME at different concentrations and the responses increased when the concentration of LIME varied from 0.003 to 30 ng · μL^{-1} with the exception of 300 ng · μL^{-1}, and most of the EAG values of different (R)-(-)-linalool treatments were negative or very low, which indicated that *H. parallela* could not be attracted by (R)-(-)-linalool alone.

Table 2 The EAG response of *Holotrichia parallela* to L-isoleucine methyl ester and (R)-(-)-linalool

Treatments (ng/μL)	EAG value (mV)	
	L-isoleucine methyl ester	(R)-(-)-linalool
0.003	2.568 ±0.613 cdBC	−0.423 ±0.571 aA
0.03	3.561 ±0.354 bcdBC	−1.072 ±0.277 cdBC
0.3	3.940 ±0.644 bcABC	−1.259 ±0.567 dC
3	4.645 ±0.725 abAB	0.616 ±0.358 dC
30	6.046 ±0.429 aA	1.384 ±0.288 bcAB
300	2.143 ±0.200 dC	–

"–": Not detected.

3.3 The EAG response of *Holotrichia parallela* to different ratios of sex pheromone compounds

The value of EAG response reached the maximum when the ratio of LIME to linalool was 7∶1, which was significantly higher than those to other ratios, and was nearly equal to the value of female sex gland supernatant. At the same time, the value in 7∶1 treatment was higher than LIME alone. This showed that linalool can enhance the attraction ability of LIME (Table 3).

Table 3 The EAG response of *Holotrichia parallela* to different ratios of sex pheromone compounds

Treatments (ng/μL)	EAG value (mV)
3∶1	1.323 ±0.416 eE
4∶1	4.519 ±0.746 dD
5∶1	5.599 ±0.634 cdCD
6∶1	7.138 ±0.366 bcBC
7∶1	11.290 ±0.807 aA
8∶1	8.818 ±0.497 bB
Crude extract	11.720 ±0.715 aA

3.4 The EAG response of *Holotrichia parallela* to sex pheromone at different concentrations

The EAG values of the mixture at different concentrations [LIME∶(R)-(-)-linalool] were measured (Table 4). The results indicated that the value increased with the concentration of the mixture ranging from 0.003 to 30 ng/μL positively, and reached the maximum when the LIME concentration was 30 ng/μL. However, the value decreased when the concentration was 300 ng/μL.

Table 4 The EAG response of *Holotrichia parallela* to sex pheromone at different concentrations

Concentration (ng/μL)	EAG value (mV)
0.003	3.514 ±0.906 cCD
0.03	3.205 ±0.432 cD
0.3	6.019 ±0.520 bBC
3	6.839 ±0.370 bB
30	15.829 ±0.389 aA
300	2.748 ±0.850 cD

3.5 Olfactory response of *Holotrichia parallela* to different ratios of sex pheromone

As shown in Table 5, most of the response rates of beetles are larger than 90%, and they had no significant difference in all the treatments, which indicated that the beetles fit for the experiment, and the rate of selective response reaches the maximum with the ratio of 7∶1 [LIME∶(R)-(-)-linalool]. Further, the results of selection coefficient showed that all treatments could attract beetles, especially the

Table 5 Olfactory response of *Holotrichia parallela* to different ratios of sex pheromones

Treatments	Response rate	Rate of selective response	Selection coefficient
3:1	0.9 ±0.173 aA	0.608 ±0.014 cbCD	0.217 ±0.029 cCD
4:1	0.9 ±0.100 aA	0.589 ±0.084 cCD	0.178 ±0.168 cCD
5:1	0.833 ± 0.058 aA	0.681 ±0.064 bcBC	0.328 ±0.075 cBC
6:1	0.9 ±0.111 aA	0.926 ±0.064 aA	0.852 ±0.128 aA
7:1	0.933 ±0.058 aA	0.967 ±0.058 aA	0.933 ±0.116 aA
8:1	0.967 ±0.058 aA	0.759 ±0.053 bB	0.519 ±0.105 bB
LIME	0.933 ±0.116 aA	0.65 ±0.087 bcBC	0.267 ±0.116 cBC
(R)-(-)-linalool	0.967 ±0.058 aA	0.482 ±0.032 dD	−0.037 ±0.0646 dD

treatment of 7∶1 [LIME∶(R)-(-)-linalool] with the selection coefficient of 0.9333. For LIME or linalool alone, the results showed lower or no attraction to the beetle.

4 DISCUSSION

Up to now, there were few researches on chemical substances of beetle. Due to the lamellate and short antennae, the EAG value reported previously was very low. According to Sun's report, the antennal sensilla of beetles locate on the inner-surface of lamellae (Sun *et al.*, 2007). In our study, we also found that it would be easy for baetles to receive the sex pheromone when the lamellate antennae of live beetle were separated from each other. Because the lamellae closed automatically when the antennae were cut from the body, it was critical that lamellae should be exposed fully in EAG experiment in theory. Our results confirmed that the EAG response was the strongest when the lamellae were separated from each other. This method established here will supply a good base for the following researches.

The EAG results showed that linalool alone had no obvious effect on EAG response but LIME could enhance EAG response. Among all of the ratios and concentrations, the response was the strongest with the mixture concentration of 30 ng · μL^{-1} and ratio of 7∶1 [LIME∶(R)-(-)-linalool], and nearly equal to that of sex gland supernatant, while the EAG value decreased when the concentration of LIME or mixture was 300 ng · μL^{-1}. These results indicate that the sex pheromone response system of *H. parallela* is very complicated and too high concentration of sex lure maybe affects the binding of its molecular to olfactory binding protein of *H. parallela*, as found in some other insects. It was reported that there are high and low limit values for the concentration of sex lure to lead to EAG response, and the concentrations of sex lure should be higher than the low limit value so that the EAG response could be recorded, but the EAG value will increase slowly when the concentration of sex lure overpasses some special value. Some reports showed that the instar of the insects would affect their EAG response since no-effect sensors increase with the insect age increasing or nerve system could decrease the sensor capability, so the EAG response will decrease when the insects grow up (Liu *et al.*, 2007; Xiang *et al.*, 2008). Here we just supplied a easy method to carried out EAG and will be helpful to study the composition of sex pheromone of *H. parallela*, but the type of sensor cell should be identified in the future work by the method of single neuronal recording.

In olfactory experiment, the selection coefficient reach the highest (0.9333) with the ratio of 7∶1 [LIME∶(R)-(-)-linalool], which showed high attraction ability, and the result is consistent with EAG results. But our results differed from the ratio of 5∶1 in *H. parallela* reported by Leal (1992) and Leal *et al.* (1993), and this may be the reason that the effect of 5∶1 mixture used in fields in China is not so good. We will study the attraction of the mixture with the ratio of 7∶1 further in fields.

ACKNOWLEDGEMENTS We would like to thank Professor Li Guo-Qing of Nanjing Agricultural University and Ph. D. Xu Xin-Jun of Shandong Entry-Exit Inspection and Quarantine Bureau for their kind help in this work.

References

Dong SL, Du JW, 2002. Identification of the sex pheromone of *Spodoptera exigua* and application in pest control. *Entomological Knowledge*, 39(6): 412 – 416. [董双林, 杜家纬, 2002. 甜菜夜蛾性信息素鉴定及应用研究进展. 昆虫知识, 39(6): 412 – 416]

Leal WS, 1992. An amino acid derivative as the sex pheromone of a scarab beetle. *Naturwissenschaften*, 79: 184 – 185.

Leal WS, Sawada M, Matsuyama S, Kuwahara Y, Hasegawa M, 1993. Unusual periodicity of sex pheromone production in the large black chafer – *Holotrichia parallela*. *Journal of Chemical Ecology*, 19(7): 1 381 – 1 391.

Liu L, Ge F, Su JW, Qin SW, Gao BJ, 2007. Electroantennogram responses of female *Dendrolimus tabulaeformis* Tsai *et* Liu to two volatile enantiomers of *Pinus tabulaeformis* Carr. *Acta Entomologica Sinica*, 50(8): 858 - 862. [刘玲, 戈峰, 苏建伟, 秦晓薇, 高宝嘉, 2007. 油松毛虫雌蛾对油松松针两种手性化合物的触角电位反应. 昆虫学报, 50(8): 858 - 862]

Sun F, Wang GL, Zhao KJ, 2007. Olfactory receptor and mechanism in scarab beetles. *Journal of Northeast Agricultural University*, 3(38): 411 - 413. [孙凡, 王广利, 赵奎军, 2007. 金龟子嗅感器及嗅觉机理的研究进展. 东北农业大学学报, 3(38): 411 - 413]

Xiang YY, Yang MF, Cui W, Lou YG, Tang Y, Li ZZ, 2008. EAG response of the male black cutworm moth, *Agrotis ypsilon* (Rottemberg) (Lepidoptera: Noctuidae) to the female's sex pheromone. *Acta Entomologica Sinica*, 51(1): 91 - 95. [向玉勇, 杨茂发, 崔巍, 娄永根, 唐毅, 李子忠, 2008. 小地老虎雄蛾对雌蛾性信息素的 EAG 反应. 昆虫学报, 51(1): 91 - 95]

Yao YS, 2004. Preliminary Study on the Positively Taxis of *Anomala Corpulenta* to Castor Bean and Its Female Sex Pheromone. MSc Thesis of Henan Agricultural University, Zhengzhou. [姚永生, 2004. 铜绿丽金龟对蓖麻的趋向作用及其雌性性信息素的初步研究. 郑州: 河南农业大学硕士学位论文]

Yu HX, 2007. The study on occurrence and control technique of *Holotrichia parallela* in peanuts. *Technology of Planting and Breeding*, (1): 51. [于海霞, 2007. 花生蛴螬的发生与防治技术研究. 种养技术, (1): 51]

Zhang Y, Liu JZ, 2003. The application of insect sex pheromone. *Bulletin of Biology*, 12(38): 7 - 10. [张岩, 刘敬泽, 2003. 昆虫的性信息素及其应用. 生物学通报, 12(38): 7 - 10]

Zhang YL, Yuan YH, Yuan GH, Guo XR, Luo MH, 2006. A study on the attraction of *Holotrichia oblita* (Faldermann) to castor leaves. *Journal of Henan Agricultural University*, 40(1): 53 - 57. [张艳玲, 袁蛮华, 原国辉, 郭线茹, 罗梅浩, 2006. 蓖麻叶对华北大黑鳃金龟引诱作用的研究. 河南农业大学学报, 40(1): 53 - 57]

暗黑鳃金龟对性信息素的触角电生理及行为反应

周丽梅[2], 鞠　倩[1], 曲明静[1,*], 赵志强[1], 董双林[3], 韩召军[3], 禹山林[1]

(1. 山东省花生研究所, 山东青岛　266100; 2. 云南农业大学植物保护学院, 昆明　650201;
3. 南京农业大学植物保护学院, 南京　210095)

摘要: 为了分离鉴定暗黑鳃金龟的性信息素成分并对其功能进行验证,本研究对比摸索了 3 种触角的处理方法,并进行同一浓度(30 ng·μL^{-1})二元混合物、不同配比的触角 EAG 测试。结果包括: 建立了一种鳃叶状触角的触角电位(EAG)测定方法,即将暗黑鳃金龟 *Holotrichia parallela* 触角的各鳃叶用针分离开后进行测定,这种方法测得的触角电位反应值较高。雄虫触角对 L-异亮氨酸甲酯和(R)-(-)-芳樟醇为 7:1 的二元混合物的反应值最高,和暗黑鳃金龟雌虫性信息素 25 个腺体提取液的 EAG 反应相当;对同一配比(7:1)不同剂量刺激液的 EAG 测试表明,雄虫对浓度为 30 ng·μL^{-1}的二元混合物刺激液的反应值最高。嗅觉反应结果进一步证实,试虫对 L-异亮氨酸甲酯和(R)-(-)-芳樟醇 7:1 的选择最高,选择系数达 93.3%。研究结果为利用性信息素防治暗黑鳃金龟技术的开发提供了基础。

关键词: 暗黑鳃金龟; 性信息素; 触角电位; 嗅觉反应; L-异亮氨酸甲酯; (R)-(-)-芳樟醇

中图分类号: Q968　　**文献标识码:** A　　**文章编号:** 0454-6296(2009)02-121-05

(责任编辑: 赵利辉)